FINAL REPORT

"Control of Biofouling Using Biodegradable Natural Products"

SERDP Project Number: PP1277

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Abstract:

Evaluated here are the inhibitory effects on the known film-forming bacteria *Pseudomonas aeruginosa* by selected plant extracts and related compounds. Tested were several members of the cinnamic acid group (caffeic, sinapic, 3-hydoxy-4-methoxycinnamic, trans-2,3-dimethoxycinnamic, 2,3-dimethoxycinnamic, and 3,4-dimethoxycinnamic acids), nicotinic and related acids (nicotinic, 2-hydroxynicotinic, 6-hydroxynicotinic, isonicotinic, picolinic, 3-hyroxypicolinic, 6-hydroxypicolinic, and citrazinic acids), benzoic acid derivatives (protocatechuic, gallic, syringic, 3-hydroxy-4-methoxybenzoic, and coumalic acids, 2,4- dihydroxybenzaldehyde, and 3,4,5-trimethoxybenzaldehyde), miscellaneous phenolics (phloroglucinol, hydroxyhydroquinone, rhodizonic acid, eugenol), and selected plant extracts (quillaja, neem, grapefruit seed extract, yucca, pinenes and limonenes). Testing was performed to determine rapid toxicity to Pseudomonas and to determine the lowest observable effective concentration of additive. The family of nicotinic acid derivatives showed significant inhibition of growth of the selected organisms at concentrations suitable for environmental use. One compound in particular, citrazinic acid showed exceptional growth inhibition, and was tested further using *Klebsiella pneumoniae* and a mixed culture. Based on the results of these tests, this compound has been identified as a potential candidate for use in heat transfer equipment systems.

Background:

Biological fouling of seawater piping and heat exchange equipment impacts nearly all oceangoing vessels, both commercial and military, resulting in poor performance and excessive maintenance costs. The current approach used by the U.S. Navy for the control of biofouling is electrolytic chlorination of seawater. Chlorine is a very effective biocide, and has been shown to be quite effective in the control of biological growth in shipboard systems. However, to achieve the desired level of performance in these applications, it is necessary to overchlorinate the water, resulting in a release of free chlorine to the ocean. At present, the Clean Water Act and 40 CFR 131 limits the amount of chlorine which can be released within 200 nautical miles the US at 7.5 ppb (chronic) and 13 ppb (acute)[1]. The CWA also gives individual states the authority to designate discharge standards applicable to 3 nautical miles, which in many cases are much more stringent than the national guidelines. In addition, the proposed Uniform National Discharge Standards (UNDS) regulations may impose restrictions on chlorine release in areas within 12 nautical miles of the United States.

The U.S. Navy has established a level of 200 ppb for 2 hours per day as the minimum chlorine level required to efficiently control biofouling in heat exchangers and condensers[2], which is well above the stated limits listed above. In order to comply with these requirements, and those which may be imposed in the future, it has become necessary to look to alternative methods for the prevention or control of biofouling.

There are many different approaches which may be considered, many of which have already been tested and reported. These approaches can be broadly categorized into three general areas: 1) antifouling coatings/paints; and 2) physical treatments; and 3) chemical treatments. There has been extensive research performed in the area of antifouling coatings with some successes (Intersleek), and much research is still ongoing in this area. These coatings provide great promise for use in hull coatings, however their use in heat exchange systems would be not be as beneficial owing to several factors including the cost of coating excessive quantities of tubing, high maintenance costs, and the decrease in thermal conductivity of metals upon coating.

Physical methods including the use of ultrasonic inducers, inserts, vortice-inducers, and plugs have been tested with poor results. Recent work involving the use of acoustics for control of biofouling is underway and shows some promise. However, concerns have been raised over the use of this technique in submarine applications.

A full spectrum of chemical treatments has been tested, and several have proven to be effective in the control of biofouling. Electrolytic chlorination of seawater falls into this category, since the effective biocide in the process is chlorine. Numerous other chemical biocides are available, however these materials either are metal-containing (tin, copper, zinc), non-biodegradable, or difficult/costly to use (peroxides, ozone). In addition, the potential of unintentional negative impact on non-targeted species is of concern. It is only recently that interest in the use of natural organic products for

antimicrobial uses has emerged, possibly due to the tightening of environmental regulations governing the use of traditional antifouling materials.

Natural plant isolates and related compounds are generally non-toxic, biodegradable, and in many cases substances contained in them are necessary for required metabolic functions. Three selected candidate plant extracts (Commercial grapefruit seed extract, Yucca shidigera, Quillaja saponaria) were examined for their effectiveness in preventing biofouling of designated metallic materials in seawater systems. Each has been reported to possess antimicrobial properties, however none has been tested for this specific application.

The testing of these additives for use in shipboard heat exchange systems requires careful consideration of the conditions which may be present. There are a number of variables to be considered including: seasonal weather changes (ambient water temperature and quantity/type of sedimentation in influent), geographical location (seawater pH, water quality, local speciation), stagnation (in-port, dead-end water mains), effects on non-metallic parts (gaskets, seals), flow rates, temperature flux, and residence time in the system. At the operational flow rate typically found in these systems (12 ft/sec), the only type of fouling which should take place is in the active piping is microfouling, primarily bacterial slime. However, since there are areas of piping which are inert, and only see occasional use (fire mains) and since even the primary systems are non-operational for extended periods of time while in port, there is ample opportunity for macrofouling to occur. By testing the additives at differing flow rates, both extremes can be modeled, and the effectiveness of the additives can be determined at any desired set of conditions by control of any of the reaction variables (pH, temperature, turbidity, etc.,).

Experimental:

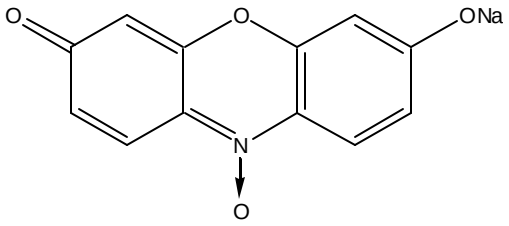
Bacteria:

Pseudomonas aeruginosa (ATCC 9027) was chosen as the primary organism of focus for testing based on its extraordinary metabolic versatility. *Pseudomonads* are adaptive microorganisms which can exist in a variety of environments. They are commonly isolated from hydraulic system biofilms and have been recognized as early colonizers in waste water systems. *Pseudomonas aeruginosa* is a Gram-negative, aerobic rod, belonging to the bacterial family *Pseudomonadaceae*. It is such a hardy species that it is often observed growing in distilled water, evidence of its minimal nutritional requirements. *Pseudomonas aeruginosa* grows optimally at 37^{o}C, but may grow in temperatures ranging from 30 – 42^{o}C. It is resistant to moderate changes in pH, and is reportedly quite resistant to antibiotic treatment. *Pseudomonas* bacteria in nature are found in either of two forms: 1) as a biofilm, whereby the bacteria are attached to a substrate; or 2) in a planktonic form, as motile cells propelled my means of flagella.

A secondary test organism, *Klebsiella pneumoniae* (ATCC 10031), was also used in some instances to provide a basis for comparison of toxic effects. *Klebsiella pneumoniae* is an aerobic, non-motile, Gram negative rod belonging to the bacterial family *Enterobacteriaceae*. One noticeable characteristic of *Klebsiella* is the presence of a large polysaccharide capsule which acts as a barrier to physical and chemical threats. Klebsiella is found in nature in biofilms, and displays many of the same properties as Pseudomonas. It is a highly adaptable organism, capable of surviving in a wide variety of environments, and is also resistant to antibiotic treatment.

Ecotoxicity tests:

Toxicities were evaluated using the commercially available ToxTrak test system (Hach, Loveland, CO, USA), method 10017. The ToxTrak test is a colorimetric test based on the reduction of resazurin, a redox-reactive dye. Upon microbial respiration, resazurin (blue) is reduced and changes color (pink). The change in absorbance is measured spectroscopically and provides for a relative determination of growth inhibition. Since there are many variables involved in this type of testing, the limits of detection are on the order of 10 percent, which correlates to the Lowest Observable Effect Concentration (LOEC). In order to determine minimum concentrations of toxins, dilutions were made until the sample was diluted sufficiently that no inhibition was observed (NOEC – No Observable Effect Concentration). It must be noted that some toxins will increase respiration and give a negative-percent inhibition on this and any other respiration-based toxicity test. Therefore, if a sample repeatedly produces results more negative than 10%, the compound is assumed to be toxic.



Resazurin: 7-hydroxy-3H-phenoxazin-3-one 10-oxide, sodium salt

Method:

Solutions of all of the selected compounds were prepared initially at a concentration of 50 parts per million (ppm). Using the ToxTrak procedure, the toxicity of these compounds to *Pseudomonas aeruginosa* (7.0×10^6 CFU) was determined. Those compounds showing significant levels of growth inhibition were selected for further testing at lower concentration levels. Testing of subsequent dilutions was performed in order to determine the NOEC level. Testing results displayed are averages of three replications.

A typical toxicity experiment was performed as follows:

1) innoculum prepared by hydration and incubation of lyophilized microorganism;
2) sample prepared using 4.5 mL biocide solution, 0.5 mL innoculum, ToxTrak reagent powder pillow, 2 drops accelerator solution;
3) initial light absorbance measured at 610 nm (control and samples);
4) light absorbance measured periodically until absorbance of control decreases 0.60 +/- .10 abs;
5) final light absorbance measured for each of the samples.

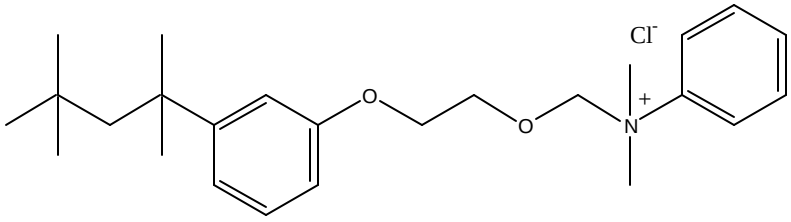
Percent inhibition is determined by the following:

$$\%I = [1-(\frac{? \text{ A sample}}{? \text{ A control}})] \times 100$$

Results and Discussion:

The primary objective of this study was to investigate the use of biodegradable, nontoxic, non-bioaccumulative materials for use as inhibitors of biological growth in shipboard heat exchange equipment. If a chemical is going to be used for this application, that chemical must be suitable for directly into the aquatic environment without producing negative effects. One way to ensure this is to use the lowest possible concentration of chemical additive. In this study, a target concentration level of 10 ppm was arbitrarily set as the maximum allowable concentration level of biocide. Restated, if an additive/extract cannot produce significant (>40%) growth inhibition of the selected microorganisms at a concentration of 10ppm, it is eliminated from further consideration.

The original proposal submitted under this SERDP Statement of Need described the investigation of three specific plant extracts, namely grapefruit seed extract (GSE), Yucca schidigera (Mojave Yucca), and Quillaja saponaria (rosacea tree). Initial testing of commercial grapefruit seed extract was initiated and looked promising, with good growth inhibition demonstrated at concentrations of 100 and 50 ppm. While this testing was being performed, a foreign literature reference was discovered[3] that suggested the antimicrobial performance of commercial GSE was not due to the extract itself, but rather due to the presence of the preservative benzethonium chloride, a quaternary ammonium compound and cationic detergent which is used as a disinfectant in cleaning solutions.



Benzethonium Chloride

As a result of this publication, an investigation of commercial GSE was undertaken by the USDA Agricultural Research Service[4]. Their report states that some of the commercial extracts tested contained up to 8% benzethonium chloride. They also came to the conclusion that it was unlikely to have been produced during the processing of the extract (and was most likely added as a preservative).

In an effort to confirm this, a laboratory grapefruit seed extract was prepared. This involved the drying and grinding of grapefruit pulp and seeds, mixing with distilled

water, then distilling to give a liquid extract. After concentration and drying, a powdered extract was obtained. This powder was mixed into glycerol and used for testing. All tests using this lab extract showed no growth inhibition of *P. aeruginosa*. Testing on GSE was stopped at this point.

Testing using Yucca schidigera extract began with solutions having a concentration of 100ppm. Growth inhibition was noted for both *P. aeruginosa* (45%) and *K.pneumoniae* (40%). Subsequent testing with 10 ppm solutions provided less favorable results, showing a 14% growth inhibition of *P. aeruginosa* , and no inhibition of *K. pneumoniae*. Testing of a 5ppm solution showed no inhibition of either microorganism.

Testing of Quillaja saponaria at 100ppm showed a 28% growth inhibition of *P. aeruginosa*, and a 3% inhibition of growth of *K. pneumonia*. Although these levels of inhibition do not meet the test criteria, subsequent testing at 10ppm was performed. These tests showed no inhibition of either microoganism.

Rather than abandon the effort at this point, a broad examination scheme was adopted to investigate a wide range of plant extracts and related compounds. Several categories of compounds were examined, each having been noted for differing levels of antimicrobial activity. Many of these compounds have been identified as allelopathic chemicals. Allelopathy refers to the direct of indirect harmful effect by one plant/organism on another through the production of chemical compounds that escape into the environment. These chemicals are produced by the plant typically as secondary metabolic products, and are used for self-preservation. Categories of compounds which have members demonstrating allelopathic properties include phenolics, alkaloids, glycosides, rare amino acids, isoprenes, and terpenes.

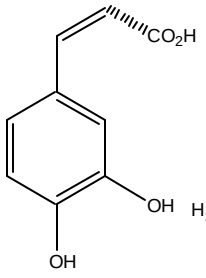
Phenolics (compounds containing a hydroxylated aromatic ring) were chosen as a major group of interest due to their abundance, relative ease of use, low cost, and history of antimicrobial performance. Quinones, phenols, and hydroxylated aromatic carboxylic acids are noted as having allelopathic properties. A well known example of this is the production of juglone (5-hydroxy-1,4-naphthoquinone) by Juglans regia (black walnut). The presence of this chemical in the proximity of the tree deters invasive plant growth and can kill plant/microbial life already established in the zone of release. Hydroxylated aromatic acids are also known fungicides. An example of this is the production of protocatechuic acid by Allium sativum (garlic). The protocatechuic acid extract is used to treat blight, mold and fungal diseases of tomatoes and potatoes.

A total of 33 compounds were examined for growth inhibition of *P. aeruginosa*. The compounds studied were placed into five groups (series) for testing. Series one consisted of six different cinnamic acid derivatives. Since this was the first test group, and no previous test results were available, the initial testing was begun at extremely high concentration levels (500 ppm). Although four of the compounds demonstrated inhibition of growth of *P. aeruginosa* at this concentration, only one (sinapic acid) was above the arbitrary 40% inhibition threshold that had been set. In an effort to better

develop the test protocol and validate the method, all of the compounds were subsequently tested at lower concentrations. At the 10ppm level, only caffeic acid and sinapic acid demonstrated any growth inhibition at all, and it was negligible. It should be noted that in many of the following cases, the respiration in a sample exceeded that of the control. In these cases the negative (-) inhibition is measured, and if it does not exceed –10%, it is assumed that the additive serves only as an additional food/energy source, increasing respiration.

		Percent Inhibition		
Series 1		500 ppm	50 ppm	10 ppm
1.	caffeic acid	25	7	0
2.	sinapic acid	42	28	0
3.	3-hydroxy-4-methoxycinnamic acid	13	17	*
4.	trans-2,3-dimethoxycinnamic acid	*	NT	NT
5.	2,5-dimethoxycinnamic acid	*	NT	NT
6.	3,4-dimethoxycinnamic acid	5	10	*

* Respiration exceeded control; NT- not tested



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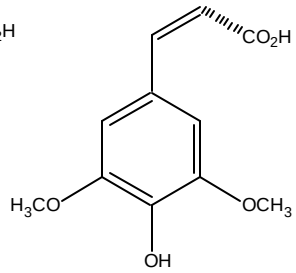

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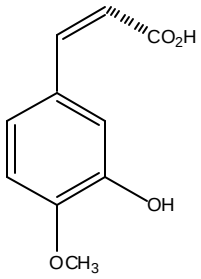

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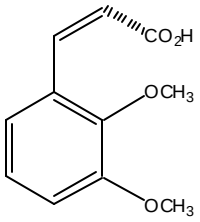

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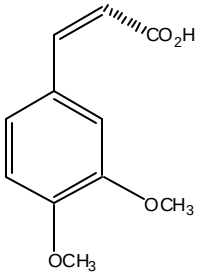

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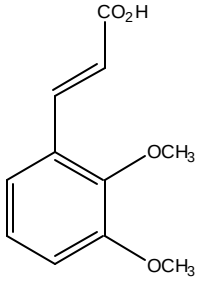

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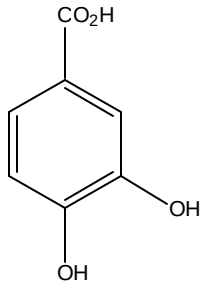
The next group of compounds examined consisted of benzoic acid derivatives. Initial testing of this group showed moderate growth inhibition at the 500 ppm concentration level for coumalic acid (44%), and minimal inhibition (<20%) for 3-hydroxy-4-methoxybenzoic acid, 2,4-dihydroxybenzaldehyde, and 3,4,5-trimethoxybenzaldehyde. For completeness, all members of the series were tested at the 10ppm level with the following results:

Series 2		% Inhibition 10ppm
1.	protocatechuic acid	*
2.	gallic acid	*
3.	syringic acid	0
4.	3-hydroxy-4-methoxybenzoic acid	2
5.	2,4-dihydroxybenzaldehyde	*
6.	3,4,5-trimethoxybenzaldehyde	*
7.	coumalic acid	10

* Respiration exceeded control

Coumalic acid was further tested at the 5ppm level inducing a six percent growth inhibition . Testing of coumalic acid at the 1ppm level showed no growth inhibition.



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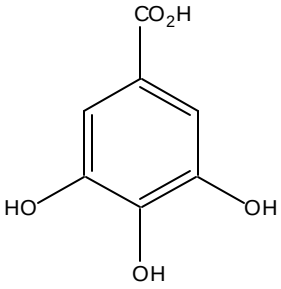

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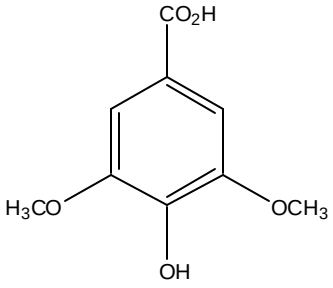

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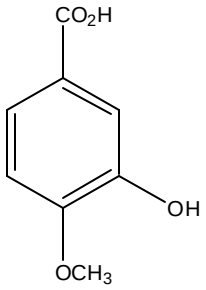

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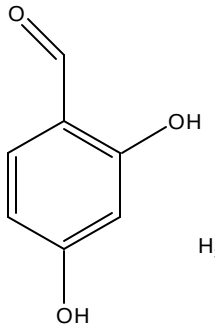

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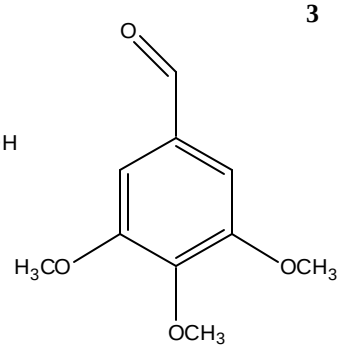

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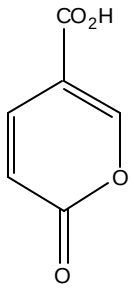

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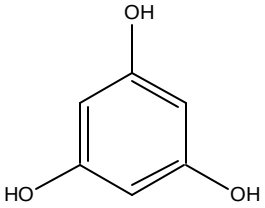
The third group of additives to be tested included several phenolic compounds along with rhodizonic acid. Of these compounds, both phloroglucinol and rhodizonic acid showed moderate growth inhibition (28%, 37%) when tested at the 500ppm concentration level.

Subsequent testing of all series members at the 10ppm level produced the following:

Series 3	% Inhibition 10ppm
1. phloroglucinol	*
2. hydroxyhydroquinone	*
3. 1,2,3-trimethoxybenzene	0
4. rhodizonic acid	31
5. eugenol	*

* Respiration exceeded control

Further testing of rhodizonic acid at the 5ppm level showed a three percent growth inhibition of *P. aeruginosa*. No further testing was pursued.



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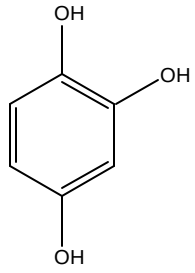

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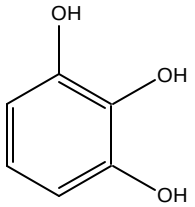

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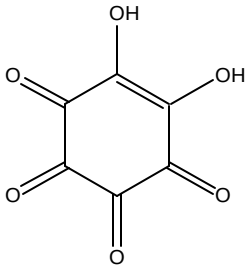

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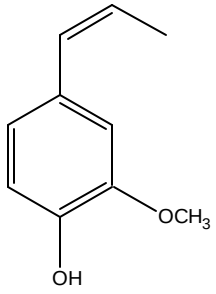

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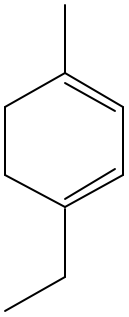
The next series of compounds examined consisted of a variety of terpenes. Initial testing at the 500ppm concentration level showed minimal growth inhibition for four of the compounds and moderate inhibition by (1R)-(+)-?-pinene. Testing of these compounds at the 10ppm level indicated that none was capable of inhibition of *P. aeruginosa*.

Series 4		Percent Inhibition 500ppm	10ppm
1.	a-terpinene	2	*
2.	myrcene	6	0
3.	(1R)-(+)-?-pinene	18	0
4.	(1S)-(-)-?-pinene	*	*
5.	(1S)-(-)-? -pinene	1	*
6.	(R)-(+)-limonene	7	*
7.	(S)-(-)-limonene	*	*



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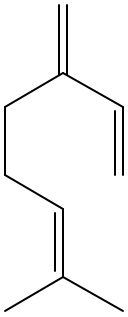

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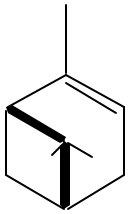

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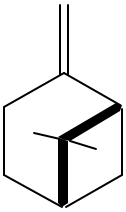

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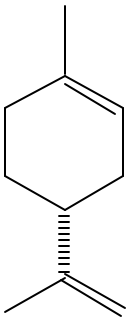
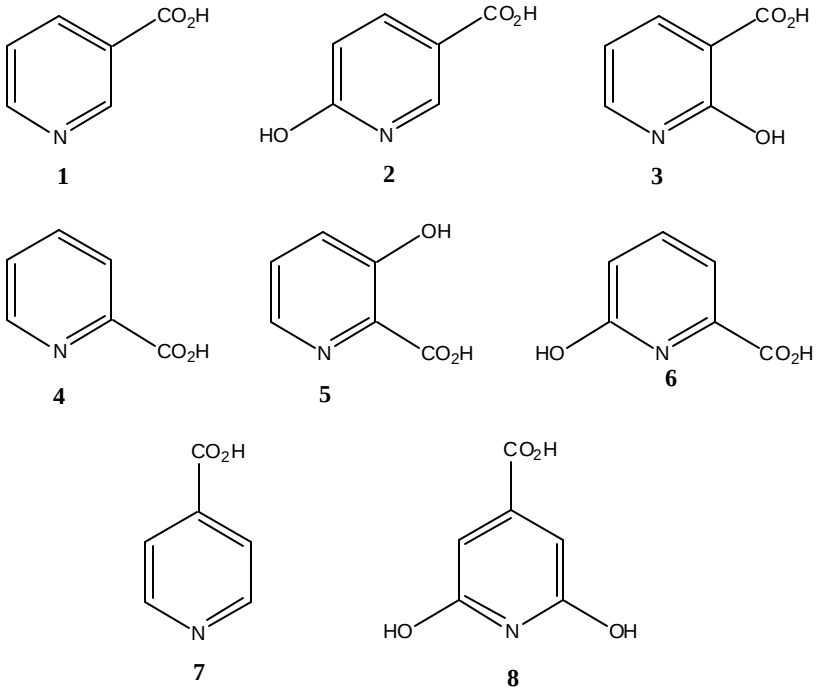


6,7

The final group of compounds tested included nicotinic acid derivatives and related compounds. These compounds all demonstrated significant growth inhibition at concentrations of as little as 50ppm. Testing of all at the 10ppm level provided the following:

Series 5		% Inhibition 10ppm
1.	nicotinic acid	6
2.	2-hydroxynicotinic acid	19
3.	6-hydroxynicotinic acid	8
4.	isonicotinic acid	24
5.	picolinic acid	11
6.	3-hydroxypicolinic acid	6
7.	6-hydroxypicolinic acid	2
8.	citrazinic acid	56



Testing of the highly inhibitive citrazinic acid at lower concentration levels was performed yielding the following results:

	Percent Inhibition				
	5 ppm	2 ppm	1 ppm	0.5 ppm	0.1 ppm
citrazinic acid	28	24	23	*	*

* Respiration exceeded control

Citrazinic acid is highly inhibitive (56%) at the 10ppm concentration level and still exhibits moderate inhibition at concentrations down to 1ppm. Based on this rapid toxicity testing, citrazinic acid was identified as the best candidate to be examined for both toxicity testing versus *K. pneumoniae*, mixed innoculum, and long term testing. Testing of citrazinic acid for growth inhibition of *K. pneumoniae* was initiated and produced the following:

	Percent Inhibition		
	10ppm	1ppm	.5ppm
citrazinic acid	26	8	*

* Respiration exceeded control

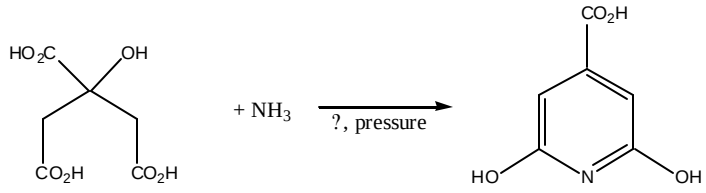
Rapid toxicity testing using a mixed culture of *P. aeruginosa* and *K. pneumoniae* provided the following results:

	Percent Inhibition		
citrazinic acid	10ppm	5ppm	1ppm
	63	48	22

Testing was undertaken to determine the long term toxicity of several members of group 5 (nicotinic acid derivatives) with respect to *P. aeruginosa*. In this experiment, turbidity measurements were used to determine relative cell populations, as the conditions were unsuitable for ToxTrak testing. Individual reaction vessels were loaded with simulated seawater (Instant Ocean, Aquarium Systems, Mentor, OH), biocide was added to achieve an overall concentration of 10ppm, and each was inoculated with *P. aeruginosa* in log phase growth. (Control used simulated sea water and innoculum only) The vessels were placed on an orbital shaker and were shaken at 100 rpm at 25^{o}C for two weeks. Initial turbidity levels were measured and recorded. Sampling was performed at regular intervals for a period of two weeks. At the end of the two week experiment, isonicotinic acid showed a 4% inhibition of growth, nicotinic acid showed no inhibition, and citrazinic acid showed a 78% percent inhibition of growth of *P. aeruginosa*. Each of the other tested compounds produced growth in excess of that of the control.

Experiments were also undertaken to determine the possible effects of citrazinic acid on stainless steel. Stainless steel coupons were suspended in solutions of varying concentrations of citrazinic acid (0-100ppm). After a period of four weeks, these coupons were removed and physically examined. There was no apparent increase in corrosion due to the presence of this additive. Longer testing times may be required to validate this statement, as well as more invasive techniques (SEM).

Citrazinic acid, although structurally related to nicotinic acid, is produced synthetically. The process involves the reaction of citric acid and aqueous ammonia under pressure, a process patented by Pfizer in the late 1950's[5].



Citrazinic acid is a yellowish powder, with a melting point > 300°C. It is soluble in alkaline and carbonate solutions, and is slightly soluble in hot acidic solution. Alkaline solutions turn a deep blue color upon extended storage. The most common use of citrazinic acid is in photographic processing, where it is used as a color developer.

Toxicity data for citrazinic acid is not abundant, but the following information is available and has been published by Kodak[6]:

Oral Rat LD50 > 3200mg/kg
Interperitoneal Rat LD50 > 800mg/kg
Skin Guinea Pig LD50 > 20mL/kg

Although data on the biodegradability of citrazinic acid is not available, information on the structurally closely related isonicotinic acid can be found. This compound is reported to completely degrade in 16-32 days in an aqueous aerobic environment, and in 32-66 days in an aqueous anaerobic environment[7]. This testing was done at a concentration of 1mmol/L, which equates to 155.1ppm, several orders of magnitude more concentrated than would be envisioned in use as a biocidal additive.

Future Plans:

There are many tests still required to determine if citrazinic acid is acceptable for use in heat exchanger applications. Most importantly, toxicity testing must be completed by a qualified toxicologist using representative aquatic indicators. Also required are mutagenicity and genotoxicity testing.

If the results of these test suites prove favorable, the next step will include testing of citrazinic acid using harbor water collected from a naval installation. These tests will be performed using a flow-through annular reactor, and will include total growth inhibition, total protein, and rate of degradation of citrazinic acid. Since actual harbor water will contain a wide variety of microorganisms, this will provide valuable insight into the potential effectiveness of this treatment in shipboard applications. Also, since the harbor water samples will most likely contains some level of heavy metals (Cu, Sn, Zn), the effects of interaction between these metals and citrazinic acid can be investigated.

While these tests are proceeding, work will begin in on the formulation of a time-release citrazinic acid composition which could then be tested in the laboratory using a flow-through system. Testing of chemical effects on metallic substrates can be performed at this time, as well as complete analysis of chemical breakdown products and bioaccumulation factors. Based on the results of these studies and with engineering consultation, a test protocol could be written and demonstration /validation of the method could be planned.

One other set of tests which should be performed is the comparison of citrazinic acid performance versus the traditional biocide chlorine. Testing could be done to determine effectiveness versus concentration of each, residuals production, and impacts to metallic substrates. The results of this would provide the direct evidence required for consideration of use in shipboard applications.

It would also be of interest to compare the results obtained using the Hach ToxTrak rapid toxicity test, to those obtained using the more well known Microtox acute toxicity test. As the Hach method is relatively new to the field, there are no published results showing comparability between the two. This would not be an expensive nor time-consuming effort, and may provide significant value to the scientific community. Since different test organisms are used in each method, it may be possible to make some generalizations regarding effectiveness of additives relative to type of organism studied.

A tentative follow-on plan would be structured as follows:

I. Toxicology studies
   a) toxicology, mutagenicity, genotoxicity
   b) toxicity testing using Microtox test

II. Testing (Harbor Water)
   a) growth inhibition
   b) rate studies/degradation
   c) interactions with metal ions

II. Formulation
   a) development of time-release composition
   b) testing of effects on metallic substrates
   c) chemical analysis of breakdown products

III. Comparative Studies (citrazinic acid vs. chlorine)
   a) minimum concentration threshold
   b) residuals production analysis
   c) comparison of effects on metallic substrates

Task	Duration	Performer	Y1-Q1	Y1-Q2	Y1-Q3	Y1--Q4	Y2-Q1	Y2-Q2	Y2-Q3	Y2-Q4
Toxicity Studies										
Tox, Mut, Gen testing	6 mo.	TBD								
Microtox testing	2 mo.	NAVAIR-CL								
GO/NO-GO DECISION										
Testing (Harbor water)	3 mo.	NAVAIR-CL								
Formulation										
Development	9 mo.	NAVAIR-CL								
Testing on metallics	12 mo.	NAVAIR-CL								
Analysis of breakdown products	3 mo.	NAVAIR-CL								
Comparative Studies										
Minimum concentration	6 mo.	NAVAIR-CL								
Residuals testing	3 mo.	NAVAIR-CL								
Comparison of metallic effects	6 mo.	NAVAIR-CL								

References:

1. "Control of Chlorine Used for Biofouling Supression in Shipboard Seawater Piping Systems"; http://p2ashore.nfesc.navy.mil

2. SEEDSON-02-06; "Environmentally Benign Biological Fouling Control in Heat Transfer Equipment"; http://www.serdp.org/seed-sons/SEEDSON-02-06.html

3. Von Woedtke, T., Schluter, B., Pflegel, P., Lindequist, U., Julich, W.D. *Pharmazie* **1999**, *54(6)*, 452-6.

4. Takeoka, G., Dao, L., Wong, R.Y., Lundin, R., Mahoney, N. *J Agric Food Chem* **2001**, *49(7)*, 3316-20.

5. US Pat. No. 2729647; US Pat. No. 2738352

6. Kodak Company Reports, 21 May, 1971.

7. Naik, M.N., Jackson, R.B., Stokes, J., Swaby, R.J. *Soil Biol. Biochem.* **1972**, *4*, 313-23.

Publications/Presentations:

1. "Control of Biofouling Using Biodegradable Natural Products", Army Industrial Ecology Information Exchange, Lake Harmony, PA, June 25-27, 2002.